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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704-0188

- **1a REPORT SECURITY CLASSIFICATION:** U
- **1b. RESTRICTIVE MARKINGS:** N/A
- **2a. SECURITY CLASSIFICATION AUTHORITY:** N/A
- **3. DISTRIBUTION/AVAILABILITY OF REPORT:** Distribution Unlimited.
- **2b. DECLASSIFICATION/DOWNGRADING SCHEDULE:** N/A
- **4. PERFORMING ORGANIZATION REPORT NUMBER(S):** Wayne State University
- **5. MONITORING ORGANIZATION REPORT NUMBER(S):** N/A

DISTRIBUTION STATEMENT A
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- **6a NAME OF PERFORMING ORGANIZATION:** Wayne State University
- **6b OFFICE SYMBOL (If applicable):** N/A
- **7a. NAME OF MONITORING ORGANIZATION:** Office of Naval Research
- **6c. ADDRESS (City, State, and ZIP Code):** Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202
- **7b. ADDRESS (City, State, and ZIP Code):** 800 N. Quincy Street, Arlington, VA 22217-5000
- **8a. NAME OF FUNDING/SPONSORING ORGANIZATION:** Office of Naval Research
- **8b. OFFICE SYMBOL (If applicable):** ONR
- **9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER:** N00014-86-K-0634
- **8c. ADDRESS (City, State, and ZIP Code):** 800 N. Quincy Street, Arlington, VA 22217-5000
- **10 SOURCE OF FUNDING NUMBERS**

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61153N	RR04108	rr1F010	

- **11 TITLE (Include Security Classification):** (U) Influence of Neuroendocrine Mediators on Phagocyte Function
- **12. PERSONAL AUTHOR(S):** Howard R. Petty
- **13a TYPE OF REPORT:** Annual
- **13b TIME COVERED:** FROM 7/1/87 TO 6/30/88
- **14. DATE OF REPORT (Year, Month, Day):** July 1, 1988
- **15 PAGE COUNT:** 14
- **16 SUPPLEMENTARY NOTATION:**

17 COSATI CODES

FIELD	GROUP	SUB-GROUP
08		

- **18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number):** Stress, Macrophage, Opioid, Adrenergic

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

This research program explores phagocyte responses to neuroendocrine mediators. We have studied the effects of epinephrine, met-enkephalin, forskolin and cAMP analogs on macrophage morphology, spreading and adherence. Cell spreading was quantitated by measuring cell perimeters. Epinephrine decreased macrophage spreading; at 10^{-5}M epinephrine the perimeter was 10.4 ± 0.3 μm in comparison to 15.0 ± 1.0 μm for controls. Epinephrine's action is blocked by propranolol. Met-enkephalin increased macrophage spreading to 18.5 ± 1.0 μm at 10^{-8}M. Since both catecholamines and opioids are released from chromaffin cells, we have examined the combined effects of both ligands. When macrophages were exposed to 10^{-5}M epinephrine and 10^{-8}M met-enkephalin, cell morphology and spreading were indistinguishable from that of epinephrine alone. The β-adrenergic receptor abrogrates the opioid signal(s). These effects may be accounted for by cAMP. Forskolin and cAMP analogs affected cell properties in the same fashion as epinephrine. Dramatic mediator-induced changes in F-actin distribution were noted.

- **20 DISTRIBUTION/AVAILABILITY OF ABSTRACT:** ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS
- **21 ABSTRACT SECURITY CLASSIFICATION:** (U)
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- **22c OFFICE SYMBOL:** ONR

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ANNUAL REPORT

Influence of Neuroendocrine Mediators on Phagocyte Function

ONR Contract No. N00014-86-K-0634

A. Introduction

In recent years it has become apparent that neuroendocrine mediators may play important roles in regulating afferent and efferent immunologic functions (1-5). The functional attributes of monocytes, macrophages, polymorphonuclear leukocytes, NK cells, T- and B-lymphocytes are modulated by neuroendocrine mediators (2,5-11). The interactive chemical communication between cells of the immunologic and neuroendocrine systems is mediated at least in part by cell surface ligand-receptor interactions. In addition to their many receptors for immunologic ligands, monocytes and macrophages have been reported to possess opiate, β-adrenergic, substance P, neurotensin, ATP, 4-carboxyglutamic acid, muscarinic and nicotinic cholinergic receptors (12-17). These neuroendocrine receptors, in concert with other immunologic or non-immunologic receptors, contribute to the regulation of macrophage function in vivo.

It is well known that certain leukocyte receptors evoke synergistic cellular responses. For example, C3b receptor ligation augments immunoglobulin Fc receptor function (18,19). However, phagocyte responses to the ligation of multiple receptors that separately trigger opposing physiologic events have not been thoroughly studied. Two in vitro models of antagonistic combinative interactions are: (1) the histamine and C3bi receptors that promote cell detachment and adherence, respectively (20), and (2) the β-adrenergic and opioid receptors that depress or enhance antibody-dependent phagocytosis (6). It is interesting to note that in both of these combinative systems, the adenylate cyclase-linked receptor abrogates the physiological effects generally ascribed to the second receptor. In the present study we have examined the individual and combinative effects of epinephrine and met-enkephalin on macrophage morphology, spreading, adherence and microfilaments. Our data: (1) provide evidence indicating that catecholamines down-regulate macrophage activities in the absence or presence of opioids and (2) suggest that elevated cAMP levels play an important role in this activity.

B. Research Results

Effects of Epinephrine, Met-Enkephalin, dbcAMP and Br-cAMP on Cell Morphology

Figure 1 illustrates the effects of epinephrine, met-enkephalin, dbcAMP, and Br-cAMP on macrophage morphology. Cells were incubated with HBSS or HBSS and mediator(s) for 30 minutes at $37^{\circ}C$. The surfaces of adherent RAW264 macrophages are relatively smooth. A representative scanning electron micrograph of cells incubated for 30 minutes at $37^{\circ}C$ with only HBSS is shown in Figure 1a. In contrast, macrophages in suspension possess many surface folds (21). Epinephrine at 10^{-5} M stimulated the formation of numerous surface folds and ridges on adherent macrophages

(Fig. 1b). However, 10^{-8} M met-enkephalin primarily induced the formation of pseudopods and membrane blebs (Fig. 1c). When both of these neuroendocrine mediators are added to cells, their appearance is similar to epinephrine-treated cells (Fig. 1d). Cells treated with dbcAMP or Br-cAMP possessed many surface folds and ruffles, similar to the results obtained with epinephrine (Fig. 1e and f).

The morphology of living macrophages was studied and quantitated by optical microscopy. In comparison to control cells (Fig. 2a), epinephrine at 10^{-5} M (Fig. 2b) did not have an apparent qualitative effect on cell morphology as judged by optical microscopy, although average cell perimeter was diminished (see below). However, 10^{-8} M met-enkephalin caused significant cytoplasmic spreading (see below) and pseudopod formation (Fig. 2c). The combined effect of simultaneous treatment with 10^{-5} M epinephrine and 10^{-8} M met-enkephalin is shown in Figure 2d. The concentrations of these neuroendocrine mediators correspond to the optimal doses found in phagocytosis and spreading assays (see ref. 6). Met-enkephalin is without effect in the presence of epinephrine. The cAMP analogs dbcAMP and Br-cAMP affected cell morphology as judged by bright-field microscopy in a fashion similar to epinephrine (data not shown).

Effects of Neuroendocrine Mediators and cAMP Analogs on Cell Spreading and Adherence

To quantitate the effects of neuroendocrine mediators and cAMP analogs on macrophage spreading, the perimeters of macrophages were measured from video images (Fig. 2). Figure 3 shows a dose-response curve illustrating the effect of epinephrine on macrophage spreading. The abscissa is the molar concentration of epinephrine while the ordinate is the percentage of control cell perimeter (the means of all experiments are compared). The measured cell perimeters decrease from 15.0 ± 1.0 μm for controls to 10.4 ± 0.3 μm for cells treated with 10^{-5} M epinephrine ($n = 4$, $P < 0.001$). The inhibitory action of epinephrine was blocked by the antagonist propranolol (see below and Fig. 7). The effect of met-enkephalin on macrophage spreading is shown in Figure 4. The measured cell perimeter increases from 15.0 ± 1.0 μm for controls to 18.5 ± 1.0 μm for cells treated with 10^{-8} M met-enkephalin ($n = 5$, $P < 0.001$). As qualitatively described by the above micrographs, met-enkephalin induces a significant increase in macrophage spreading whereas epinephrine induces a dramatic decrease in spreading.

Although the above data quantitatively describe the mean cell perimeters during several conditions, they do not illustrate the cell-to-cell variability of the populations. Figure 5 shows the distribution of cell perimeters of macrophages during several experimental conditions. These data represent a compilation of data taken during at least four different trials. Cell perimeters were divided into four groups: 32-43, 46-57, 61-71, and 75-84 μm. These groupings were chosen because they correspond to convenient map reader divisions. The number of cells from all trials within each group were added together then plotted at the ordinate. The effects of epinephrine, met-enkephalin, and the simultaneous addition of both mediators are shown in Figure 5. Although the mean cell perimeter drops 25% in the presence of 10^{-5} M epinephrine, the number of cells in the smallest perimeter group increases over 600%. Similarly, 10^{-8} M met-enkephalin increases mean cell perimeter; the enhancement is most pronounced at the highest perimeter grouping (75-84 μm). The simultaneous addition of both 10^{-5} M epinephrine and 10^{-8} M met-enkephalin led to a population distribution indistinguishable from that of epinephrine alone. The combinative effects of epinephrine and met-enkephalin returned to control levels by the addition of propranolol (Fig. 7).

The potential role of cAMP in mediating these changes in spreading behavior was tested using cAMP analogs and forskolin. Figure 6 shows the distributions of cell perimeters for macrophages exposed to 30 μM dbcAMP, 30 μM Br-cAMP, 10^{-5} M forskolin and matched controls using buffer only. Both cAMP analogs induce a substantial decrease in cell spreading. The effect of dbcAMP could not be reversed by propranolol (Fig. 7). Furthermore, forskolin, a reagent that stimulates endogenous cAMP production, leads to diminished spreading indistinuishable from that of the exogenous analogs. The cell populations of Figures 5 and 6 suggest that: (1) epinephrine screens out the spreading signal of met-enkephalin and (2) this effect may be due to cAMP.

In Figure 7 we show a summary of experiments using the antagonist propranolol. As expected, the addition of 10^{-5} M propranolol has no effect on macrophage spreading (99.5 $\pm$ 1.2% of control). In addition, propranolol could not block the ability of dbcAMP to diminish spreading. However, propranolol did inhibit the activity of epinephrine and epinephrine plus met-enkephalin on spreading. Interestingly, 10^{-5} M propranolol diminished the ability of met-enkephalin to augment spreading. The ability of propranolol to influence the activity of opioid receptors was unexpected; participatory factors may include: (1) partial agonist activity of this pharmacologic reagent, (2) a generalized membrane effect, and/or (3) receptor-receptor interactions.

The abilities of epinephrine, met-enkephalin, epinephrine plus met-enkephalin, and dbcAMP to affect RAW264 macrophage adherence were tested. Met-enkephalin at 10^{-6} and 10^{-8} M produced significant and reproducible increases in adherence of 119 $\pm$ 6.8% and 112 $\pm$ 5.0% in comparison to controls, respectively (n=4; $P<0.001$ for each). This is consistent with a recent study of neutrophil adherence by Van Epps and Kutvirt (22). Met-enkephalin at other doses and epinephrine and dbcAMP at all doses tested did not influence adherence. No significant change in adherence was found for the combinative experiment using 10^{-5} M epinephrine and 10^{-8} M met-enkephalin (n=3; 92 $\pm$ 16%).

Effects of Neuroendocrine Mediators and cAMP Analogs on Actin Filaments

The ability of epinephrine, met-enkephalin, and cAMP reagents to affect cell morphology and spreading suggests the involvement of cytoskeletal assemblies. To test the effects of these materials on the cytoskeleton, RAW264 macrophages were incubated in the absence or presence of reagents for 30 minutes at 37°C, as described above. Cells were then fixed, extracted, and stained with NBD-phallacidin. Figure 8 shows fluorescence photomicrographs of macrophages after exposure to several conditions. Control and met-enkephalin (10^{-8} M)-treated cells possessed linear and punctate fluorescent structures (Fig. 8a and c). These structures were particularly apparent in peripheral regions of the cells. In contrast, macrophages treated with epinephrine, epinephrine plus met-enkephalin, dbcAMP, or Br-cAMP demonstrated an intense cortical band of F-actin (Fig. 8b,d,e, and f). This suggests that receptor-cytoskeletal communication, possibly mediated by cAMP, leads to altered cell morphology and spreading.

C. Publications

1. H.R. Petty and K.A. Berg (1988) Combinative Ligand-Recpetor Interactions: Epinephrine Depresess RAW264 Macrophage Phagocytosis in the Absence and Presence of Met-Enkephalin. **J. Cell. Physiol.** 134, 281-286.

2. H.R. Petty and S.M. Martin (1988) Combinative Ligand-Receptor Interactions: Effects of cAMP, Epinephrine, and Met-Enkephalin on RAW264 Macrophage Morphology, Spreading, Adherence, and Microfilaments. **J. Cell. Physiol.**, submitted.

D. Awards

1. Elected as a Fellow of the American Associaiton for the Advancement of Science

2. Wayne State Fund Research Career Development Chair Award

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FIGURE LEGENDS

Fig. 1. Scanning electron micrographs of macrophages exposed to mediators or cAMP analogs for 30 minutes at 37°C are shown. Cells were prepared for electron microscopy as described in Materials and Methods. Representative micrographs of macrophages treated with: (a) buffer alone (x 2980), (b) 10^{-5} M epinephrine (x 4020), (c) 10^{-8} M met-enkephalin (x 3940), (d) 10^{-5} M epinephrine plus 10^{-8} M met-enkephalin (x 6200), (e) 30 μM dbcAMP (x 4020), and (f) 30 μM Br-cAMP (x 6200).

Fig. 2. The effects of epinephrine, met-enkephalin, dbcAMP and Br-cAMP on cell morphology are illustrated. Macrophages were allowed to adhere to coverslips for 30 minutes at 37°C in the absence or presence of these substances. Representative bright-field micrographs of macrophages treated with: (a) buffer alone, (b) 10^{-5} M epinephrine, (c) 10^{-8} M met-enkephalin, (d) 10^{-5} M epinephrine and 10^{-8} M met-enkephalin (x 1,200).

Fig. 3. A dose-response curve demonstrating the effect of epinephrine on macrophage spreading is shown. The mean cell perimeter is listed at the ordinate and the molar concentration of epinephrine is given at the abscissa. The mean ± s.e.m. of four independent trials are shown.

Fig. 4. A dose-response curve illustrating the effect of met-enkephalin on macrophage spreading is shown. The mean cell perimeter is given at the ordinate; the abscissa lists the molar concentration of met-enkephalin. The mean ± s.e.m. of four independent trials are given.

Fig. 5. Analyses of the distribution of macrophage perimeters during four sets of conditions are given. The cell perimeters are distributed between four groupings whose lengths were 32-43, 46-57, 61-71, and 75-84 μm. these groups are listed along the abscissa. The total number of cells for all trials is given at the ordinate. The population distributions of cells treated with buffer alone (———), 10^{-5} M epinephrine (— — —), 10^{-8} M met-enkephalin (· · · ·), and 10^{-5} M epinephrine + 10^{-8} M met-enkephalin (_ · _ ·).

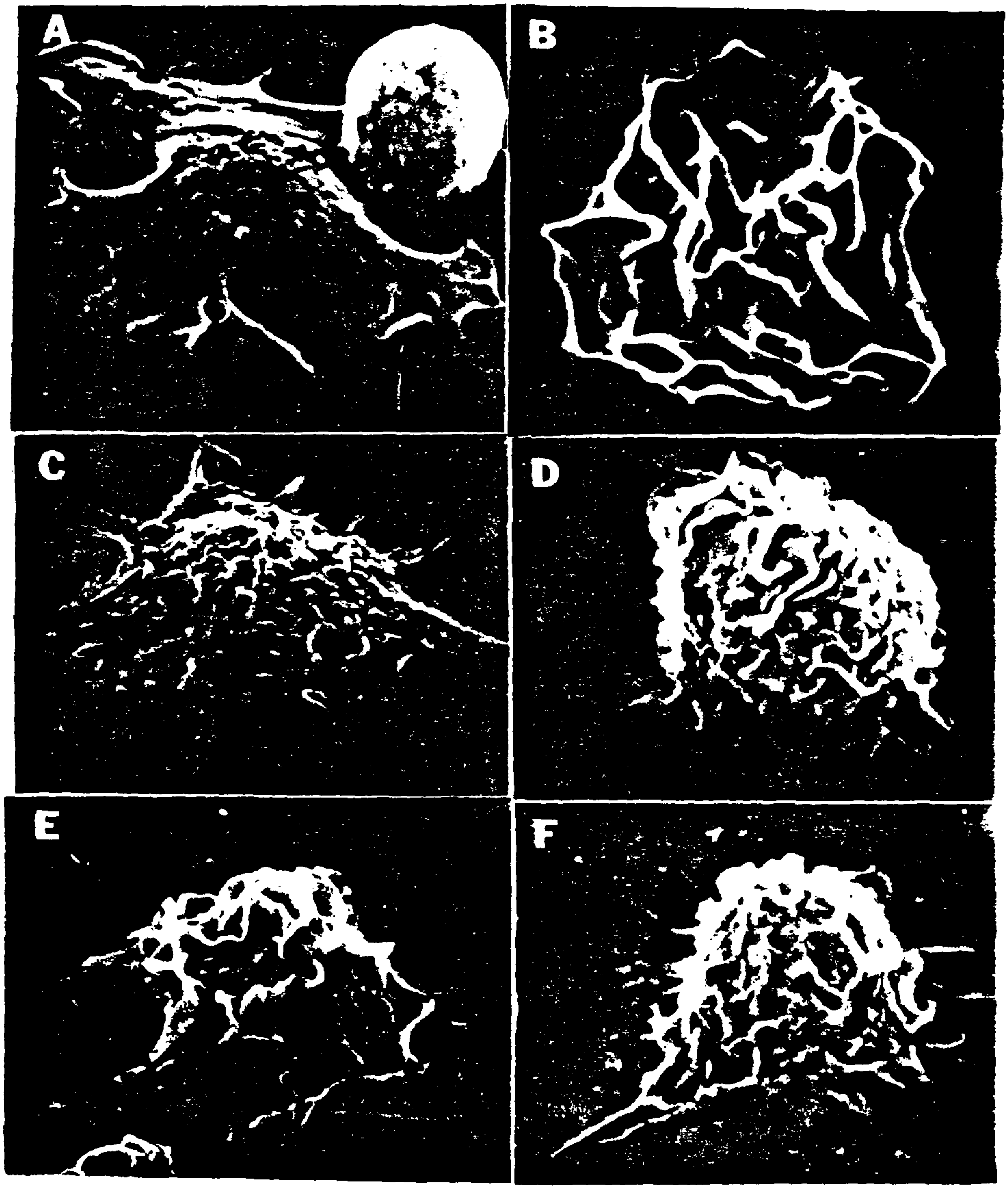
Fig. 6. The distribution of macrophage perimeters in the presence and absence of cAMP analogs is shown. Data are plotted as described in the legend of Fig. 5. The effects of buffer (———), 30 μM dbcAMP (...), 30 μM Br-cAMP (-----), and 10^{-5} M forskolin (— - —) are shown (n = 3).

Fig. 7. A bar chart listing the effects of the β-adrenergic antagonist propranolol on macrophage spreading is shown. The percent of the mean control perimeter is shown (mean ± s.e.m.). Five sample conditions are given along the abscissa. These conditions are: control, 10^{-5} M epinephrine, 10^{-8} M met-enkephalin, 10^{-5} M epinephrine plus 10^{-8} M met-enkephalin, and 30 μM dbcAMP. The open bars represent data in the absence of propranolol; the hatched bars represent identical experiments conducted

in the presence of propranolol (n = 3 to 6; control n = 26 for these trials).

Fig. 8. Fluorescence photomicrographs of RAW264 macrophages labeled with NBD-phallacidin. Cells were exposed to mediators for 30 minutes at 37°C followed by fixation, extraction, and staining. This gallery of photomicrographs includes cells treated with: (a) buffer alone, (b) 10^{-5} M epinephrine, (c) 10^{-8} M met-enkephalin, (d) 10^{-5} M epinephrine plus 10^{-8} M met-enkephalin, (e) 30 μM dbcAMP, and (f) 30 μM Br-cAMP. (x 1,000).

Figure 1



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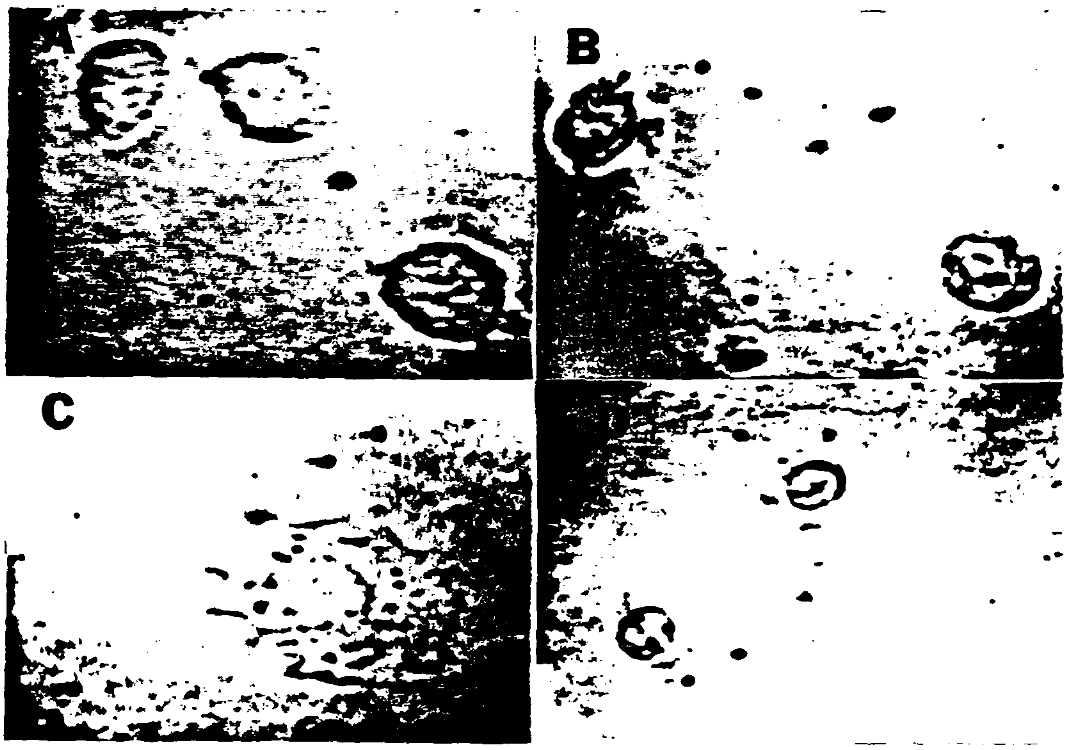

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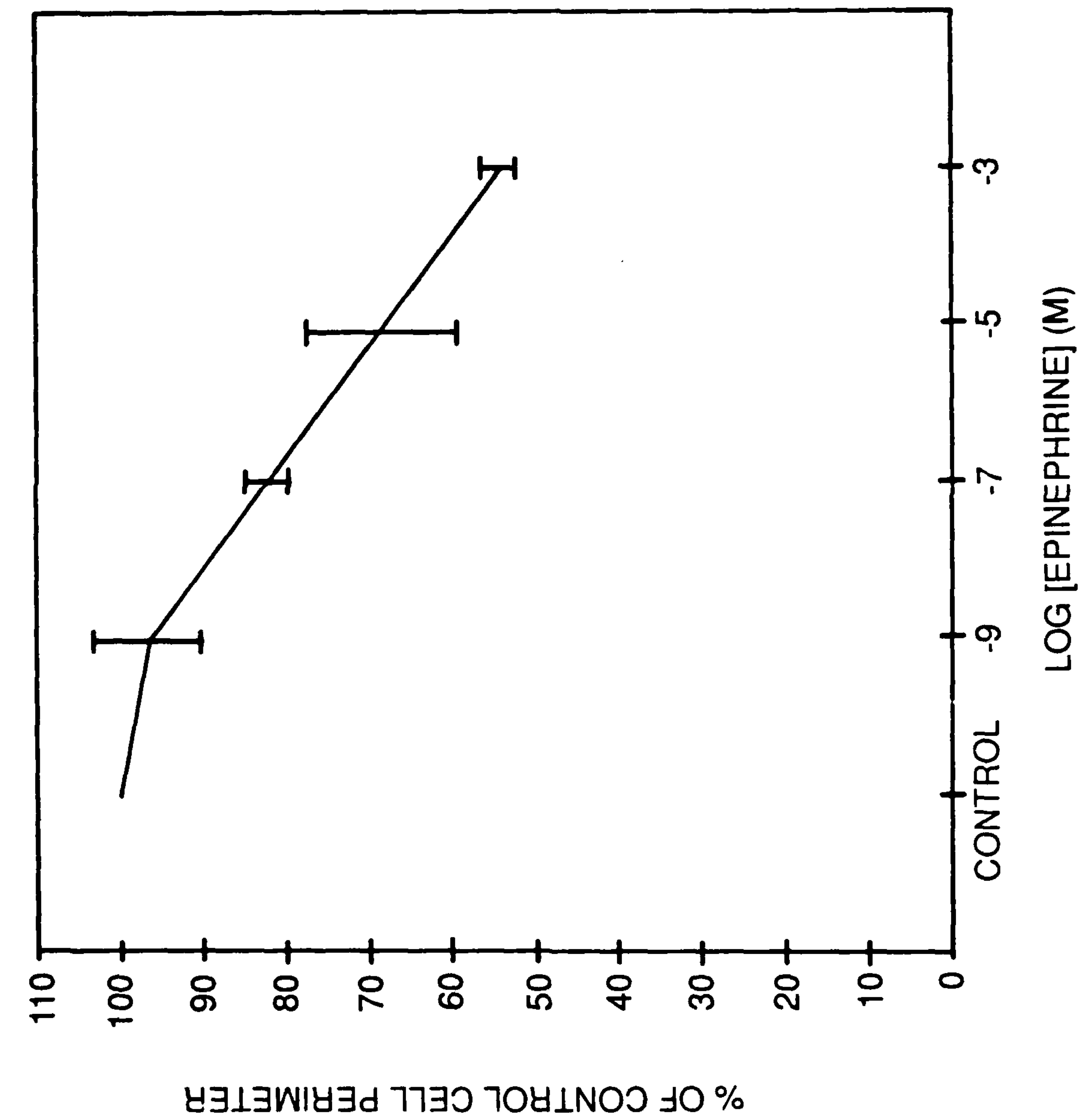
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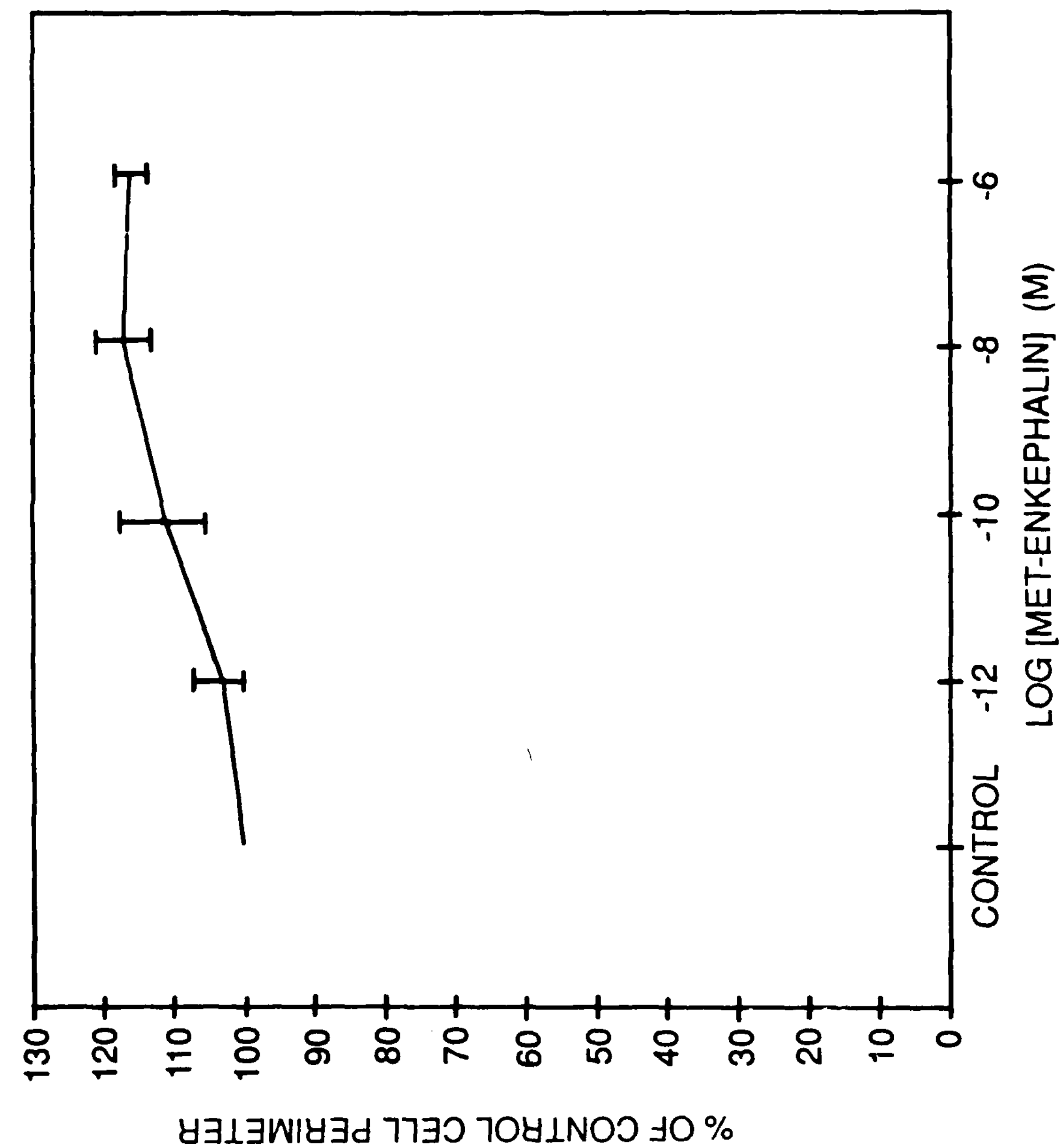
100
90
80
70
60
50
40
30
20
10
0
CONTROL
-9
-7
-5
-3
LOG [EPINEPHRINE] (M)

Figure 4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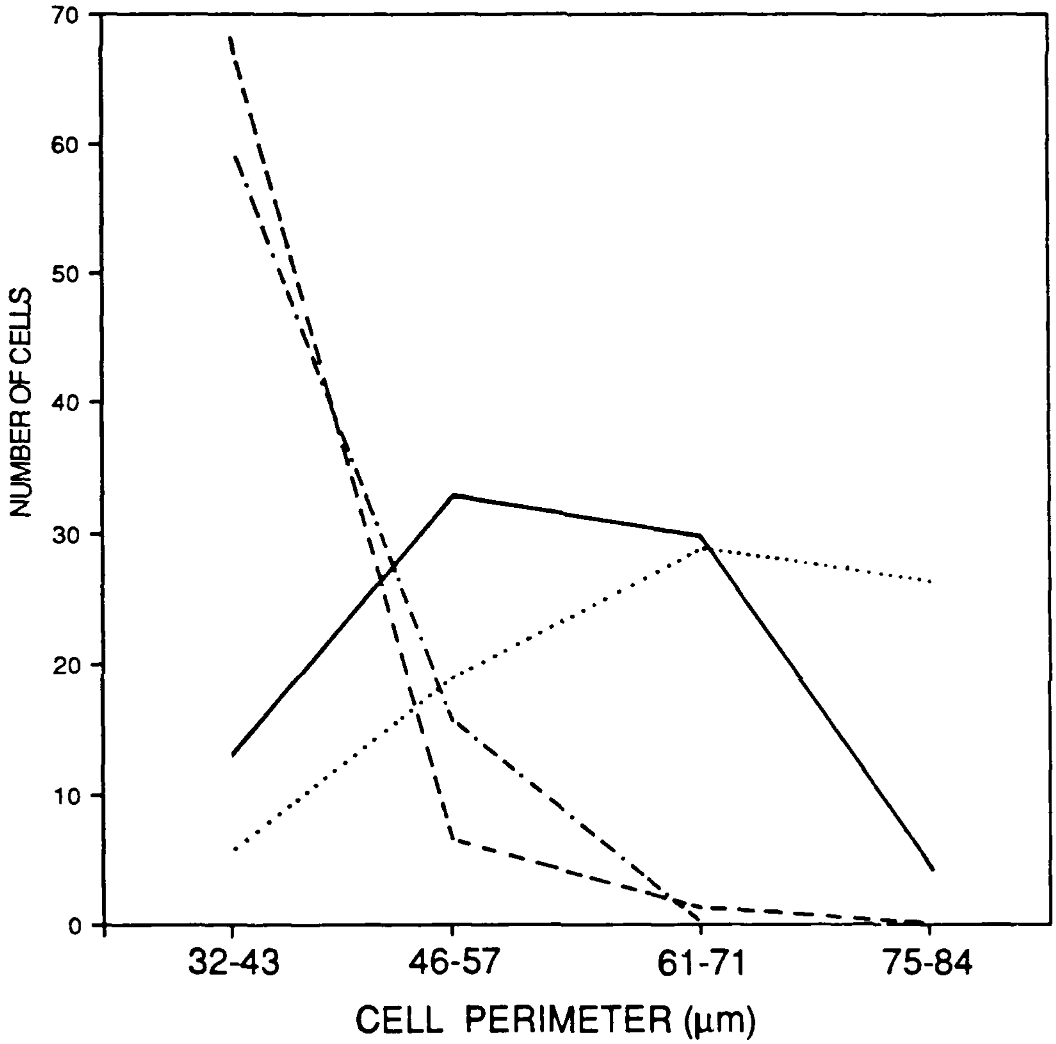
-6
LOG [MET-ENKEPHALIN] (M)

Figure 5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50
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NUMBER OF CELLS
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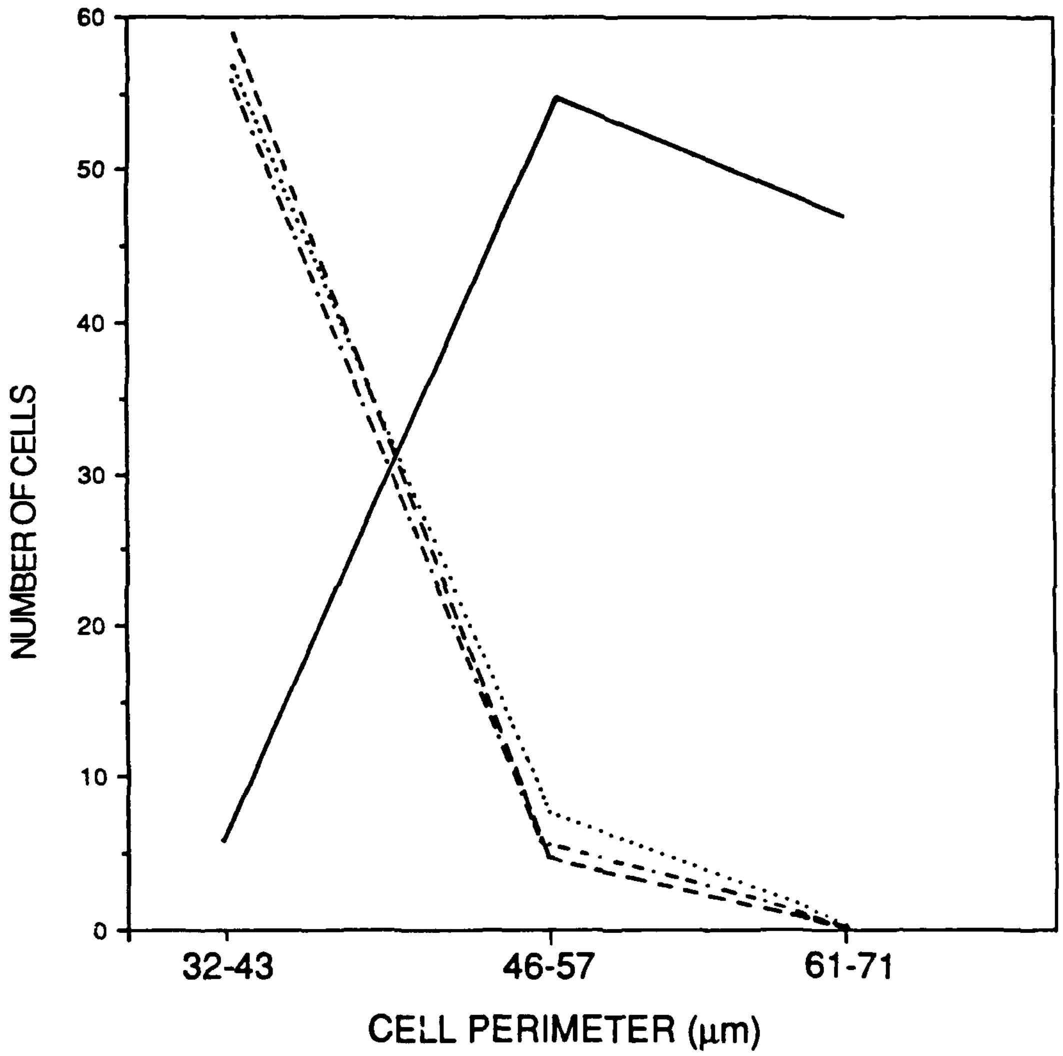
46-57
61-71
75-84
CELL PERIMETER (µm)

Figure 6



60
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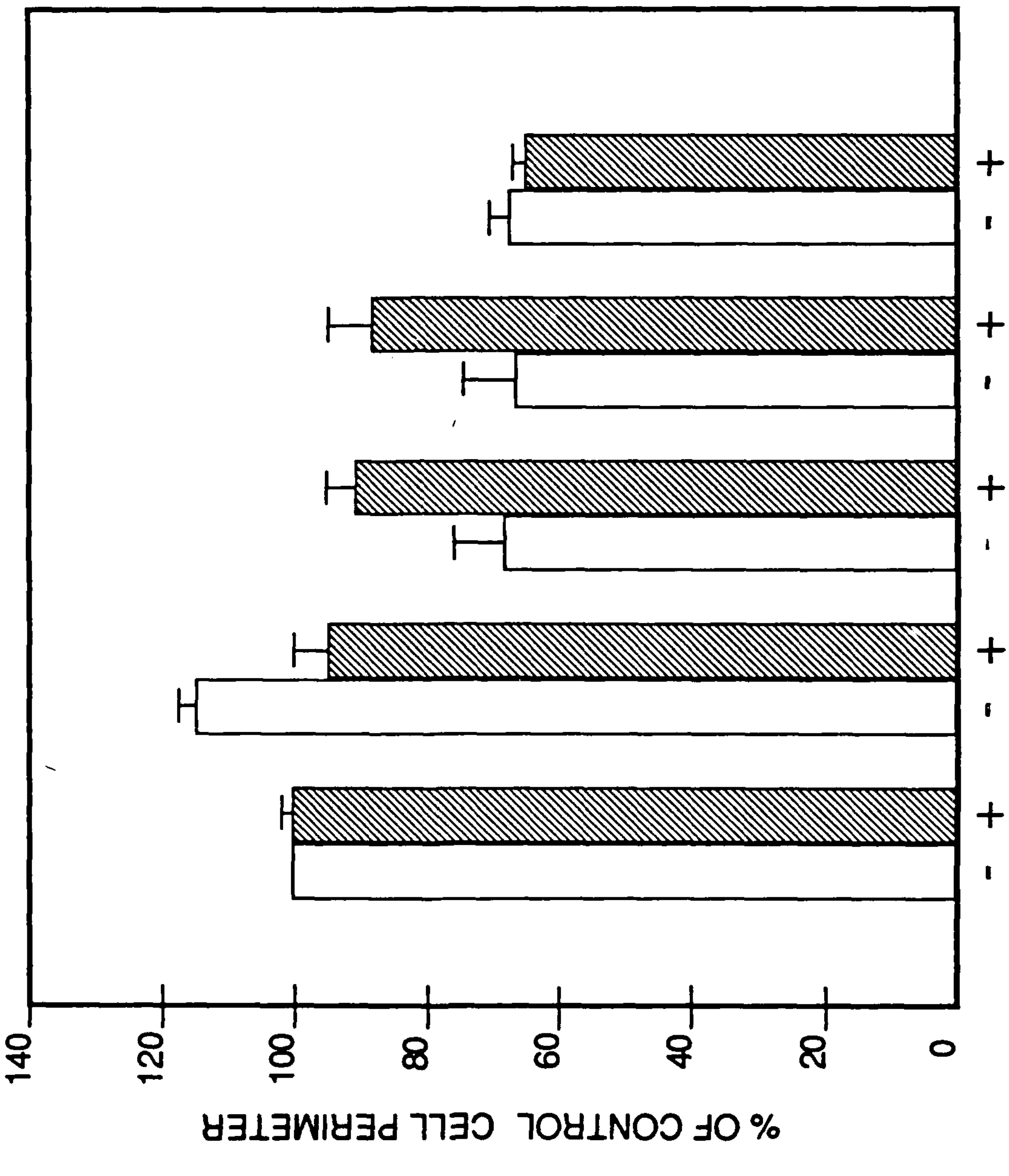
0
NUMBER OF CELLS
32-43
46-57
61-71
CELL PERIMETER (µm)

Figure 7



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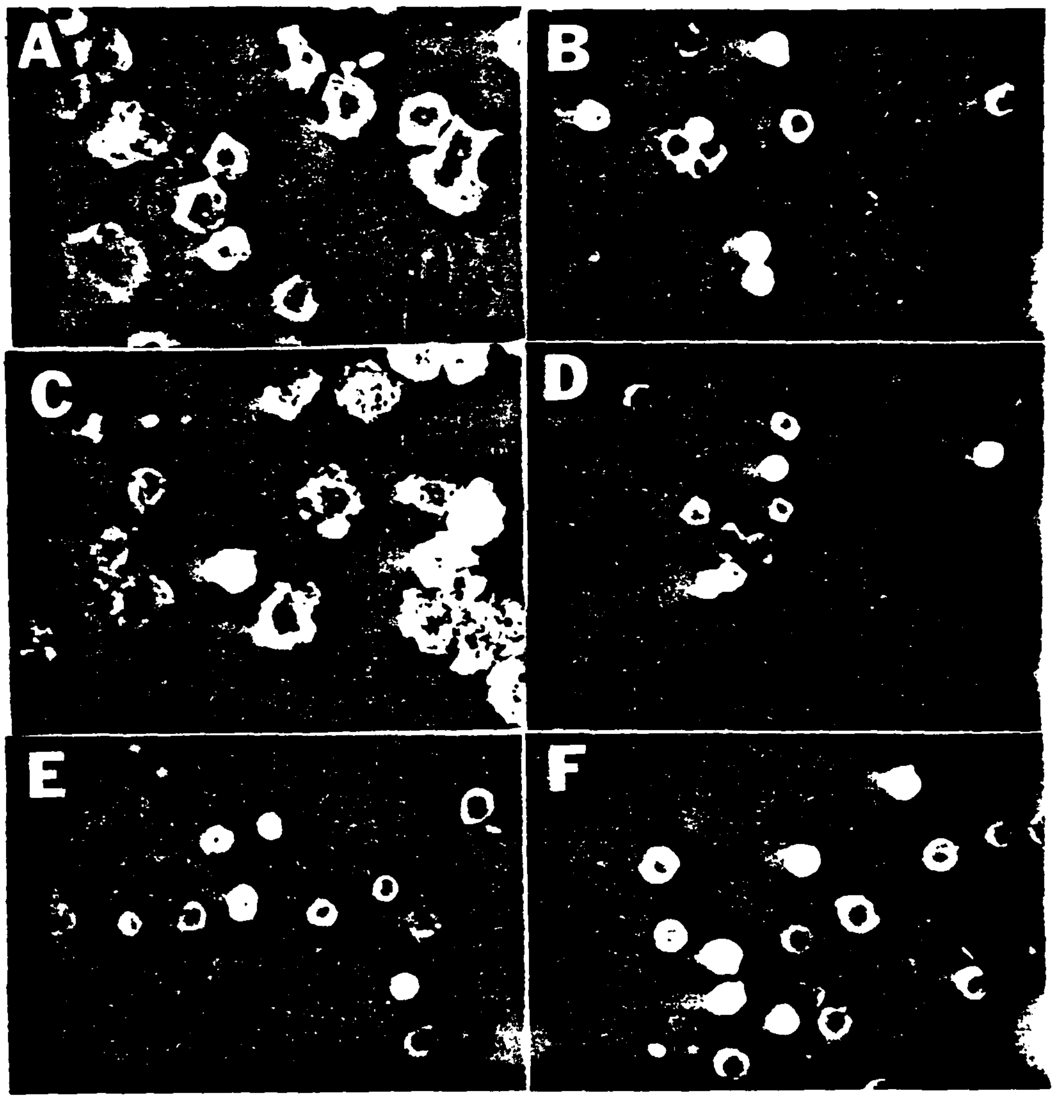

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